

GENETIC STRUCTURE AND DEMOGRAPHY OF *CHLOROPYRON PALMATUM*,
AN ENDANGERED ANNUAL PLANT

DEBRA R. AYRES¹

Evolution and Ecology, University of California, Davis, CA 95616,
¹drayres@ucdavis.edu

ERICA FLEISHMAN

John Muir Institute of the Environment, University of California, Davis, CA 95616

ALAN LAUNER

Land, Buildings, and Real Estate, Stanford University, Stanford, CA 94305

ALEX K. LEE

700 Heinz Ave., Suite 200, Berkeley, CA 94710

DAVID ZIPPIN

ICF International, 620 Folsom St., Suite 200, San Francisco, CA 94107

ABSTRACT

Chloropyron palmatum (Ferris) Tank & J.M Egger (formerly *Cordylanthus palmatus* [Ferris] J. F. Macbr.) is an annual plant that inhabits seasonally flooded wetlands with saline and alkaline soils in California. In 1986, the plant was listed as endangered under the U.S. Endangered Species Act. We aimed to inform conservation strategies for the species' five remaining populations by examining the genetic diversity and structure of the populations (on the basis of nuclear DNA markers) and their potential response to demographic and environmental stochasticity. We also assessed fluctuations in population size and whether there was evidence of hybridization between *C. palmatum* and *Chloropyron molle* (A. Gray) A. Heller subsp. *hispidum* (Pennell) Tank & J.M. Egger (formerly *Cordylanthus mollis* A. Gray subsp. *hispidus* [Pennell] T.I. Chuang & Heckard). Populations of *C. palmatum* were genetically distinct with a F_{ST} of 0.23, indicating substantial genetic structure among populations. Within populations, there was no evidence of isolation by distance. However, individuals in two adjacent vernal pools were genetically distinct. The pattern of genetic variation within populations suggests that the historical frequency and extent of seed dispersal by overland flooding has strongly affected the genetic structure of populations. Despite founder effects and population bottlenecks, small and large populations had similar levels of genetic variation. We found no evidence of hybridization. All extant populations of *C. palmatum* are genetically variable and distinct. We recommend that hydrologic connectivity be considered if seeds are collected and sowed with the intent of increasing the size of natural populations or creating experimental populations.

Key Words: alkali wetlands, *Cordylanthus palmatus*, genetic structure, hydrological connectivity, inter-simple sequence repeats (ISSRs), palmate-bracted bird's beak, vernal pools.

Declines in occupancy and abundance of most species are caused by habitat loss and reductions in habitat quality (Foin et al. 1998). Often these declines are accompanied by genetic changes, but focusing on genetics as a means to maintain or restore plant populations is relatively uncommon (Hufford and Mazer 2003). However, when habitat is highly limited and cannot be restored or reconstructed, genetics may be relevant to in situ conservation efforts such as increasing population sizes and establishing seed banks, particularly for annual plants (Ramp et al. 2006). Many plants, especially rare taxa, are associated with certain microhabitats (Maliakal-Witt et al. 2005). When these microhabitats occur in discrete, small patches, the genetics of populations and species may diverge at fine spatial resolution. If genetic data are assessed relative to potential drivers of genetic structure (i.e., the distribution

of genetic variation within and among populations), they can inform efforts to maintain or simulate ecological phenomena that affected the microevolution of the species.

Genetic structure develops over ecological and evolutionary time. When gene flow is reduced, populations can diverge in response to the random processes of mutation and drift, directional processes such as natural selection, or both. Conversely, genetic divergence can be forestalled when gene flow is frequent and widespread. By combining data on genetic structure, trends in population size, and breeding system and field observations of potential mechanisms of gene flow, one can identify biotic and abiotic drivers of genetic structure.

Chloropyron palmatum (Ferris) Tank & J.M Egger (Orobanchaceae) is an annual plant that inhabits seasonally flooded wetlands with saline

and alkaline soils in California's Central and Livermore valleys. The plant survives the dry summer through hemiparasitism (Chuang and Heckard 1971), excretion of salt crystals through its leaves, and deep rooting (USFWS 1998). The species was listed as endangered under the California and United States Endangered Species Acts in 1984 and 1986, respectively, due to the small sizes of or threats to the five remaining populations. Geographic distances among populations exceed the probable dispersal ability of the species, and virtually no other habitat remains. Accordingly, understanding of genetic structure may be a substantial asset to planning and implementation of in situ and ex situ conservation actions for the species.

Experiments in which enclosed flowers on individual plants were hand pollinated with pollen from the same plant suggested that *C. palmatum* is self-compatible (CCB 1993 unpublished data). *Chloropyron palmatum* is believed to be insect pollinated because the flowers are closed at anthesis, and therefore wind pollination is unlikely (Iwanami et al. 1988). Experiments demonstrated that neither fruit nor seed set in the absence of pollinators (CCB 1993 unpublished data). A few studies found that *Bombus californicus* Smith, 1854 and *B. vosnesenskii* Radoszkowski, 1862 pollinate *C. palmatum* (CCB 1994, Lee and Associates 2002). However, *C. palmatum*, like other plants with relatively long flowering periods, may have a succession of pollinators. In addition to *Bombus*, these pollinators appear to include solitary bees, such as *Halictus* spp. Latreille, 1804 and *Lasioglossum* spp. Curtis, 1833 (CCB 1992, Lee and Associates 2002). Scanning electron micrographs of the legs of halictid bees collected at flowers of *C. palmatum* confirmed that the bees could carry *C. palmatum* pollen (CCB 1992). Nonetheless, the low fruiting rate of plants from which *Bombus*, but not *Halictus*, experimentally were excluded suggested that the small, solitary bees did not play a substantial role in pollination (CCB 1994).

In general, bees begin to collect pollen at the lowest flower of a columnar inflorescence, such as the spike in *C. palmatum*, and move upward (Proctor and Yeo 1972). Bees then fly to inflorescences on neighboring plants, with occasional longer-distance flights. Previous observations suggested that *B. californicus* transports considerable amounts of *C. palmatum* pollen within and among plants, potentially facilitating both inbreeding and outcrossing (CCB 1993 unpublished data, CCB 1994). Further work suggested that that *Bombus* remain in a given patch of *C. palmatum* during the period in which they are foraging on that species (CCB 1994). A potential consequence of this behavior is limited transfer of pollen among patches of *C. palmatum*. If seed dispersal is limited, then

the net result of self-compatibility and limited pollen movement is development of genetic structure at a resolution commensurate with pollination distance.

The small size, crested coats, and hair-like wax extrusions of *C. palmatum* seeds (Chuang and Heckard 1972) suggest that they are dispersed by water (Howe and Smallwood 1982). A laboratory study showed that fresh seed could float well for six days (Ayres unpublished data). However, earlier field and greenhouse studies suggested that *C. palmatum* seeds also are dispersed by gravity (CCB 1993 unpublished data, CCB 1994). Because *C. palmatum* occurs in areas with anthropogenic impediments to the flow of surface water, we aimed to determine whether genetic structure within populations could be attributed to hydrologic isolation. Long-distance dispersal of buoyant seeds by surface water might prevent development of genetic structure within populations, whereas restricted surface-water movement or dispersal of seeds by gravity might lead to fine-resolution genetic structure. Patches of wetlands inhabited by *C. palmatum* include alkali or saline vernal pools of several hundred square meters and an alkali sink with dozens of connected and isolated vernal pools and swales.

A previous genetic study of *C. palmatum* based on six polymorphic enzyme loci found that approximately 98% of genetic variation was contained within populations, whereas 2% of genetic variation occurred among populations (Fleishman et al. 2001). One of the five populations contained a disproportionate amount of the within-population genetic variability (Fleishman et al. 2001). Fine-grained genetic structure (less than four meters) was detected in the latter population. Because lack of isozyme polymorphism may have affected those inferences, we used many nuclear DNA loci to investigate genetic structure. *Chloropyron molle* A. Gray subsp. *hispidum* (Pennell) Tank & J.M. Egger is sympatric with the most genetically variable population of *C. palmatum*. Therefore, we also aimed to determine whether hybridization between the two species might explain increased enzyme allelic diversity in this population. The ultimate goal of our research was to inform conservation strategies for *C. palmatum* by increasing our understanding of microevolutionary processes (Ayres et al. 2007).

METHODS

Study System

We examined genetic variation and estimated abundance of all five known populations of *C. palmatum*. Climate throughout the species' range is similar. Virtually all precipitation occurs as rain during the winter, and summers are hot

and dry. *Chloropyron palmatum* grows on seasonally flooded, saline-alkali soils in lowland plains and basins. It grows primarily along the edges of channels and drainages, with a few individuals scattered in seasonally wet depressions, alkali scalds, and grassy areas. *Chloropyron palmatum* occurs with other species tolerant of high salt concentrations, such as iodine bush (*Allenrolfea occidentalis* [S. Watson] Kuntze), alkali heath (*Frankenia salina* [Molina] I.M. Johnst.), glasswort (*Arthrocnemum subterminale* [Parish] Standl.), bush seepweed (*Suaeda nigra* J.F. Macbr.), and salt grass (*Distichlis spicata* [L.]).

The Mendota population (Fresno County) is the southernmost population of *C. palmatum*. It occurs within the Alkali Sink Ecological Reserve, which covers approximately 380 ha. Within the reserve, *C. palmatum* is associated with iodine bush and other native shrubs. Host plants have not been identified conclusively, but in 1988 most *C. palmatum* grew within 2 m of bush seepweed (*Suaeda nigra*) and rusty molly (*Kochia californica* S. Watson) (E. Cypher, unpublished data).

The Livermore population (Alameda County) is located in the southern part of a ca. 7000 ha hydrologic basin, the Springtown Alkali Sink. About 300 ha of the sink, of which 109 ha are owned by the city of Livermore and designated as a preserve, are a mosaic of alkali sink shrubs and grasses, annual grasslands dominated by non-native species, natural and channelized stream drainages, and permanent and seasonal streams and vernal pools. The upper portions of the basin contain agricultural lands and annual grasslands.

The Woodland population (Yolo County) occurs on the 73 ha Alkali Grasslands Preserve, which is owned by the city of Woodland and a private citizen. The preserve is dominated by annual grasses with areas of alkali grasses, seasonal pools and swales, a vernal lake, irrigation channels, and levees. The most abundant annual grasses are non-native species, especially *Lolium perenne* L. subsp. *multiflorum* (Lam.) Husnot. The hydrology of the preserve has been highly altered by agricultural activities. In at least two years (1974 and 1984), no mature plants or seedlings of *C. palmatum* were observed (M.A. Showers, personal observations); the population likely reestablished from the soil seed bank. Within the preserve, *C. palmatum* occurs in two locations separated by ca. 1.6 km but connected by seasonal drainages.

The Colusa population (Colusa County) is located within the 1,872 ha Colusa National Wildlife Refuge. Colusa National Wildlife Refuge lies within the Colusa Basin and historically was covered by alkali vernal pools and associated alkali meadows. Prior to its designation as a refuge, the land was used for livestock grazing and for growth of winter wheat and rice. Parts of the refuge subsequently have reformed into

functional vernal pools and alkali meadows. We sampled *C. palmatum* from two adjacent vernal pools.

The Delevan National Wildlife Refuge (Colusa County) includes more than 1820 ha of intensively managed wetlands and 486 ha of uplands. *Chloropyron palmatum* occurs primarily in alkali meadows and large vernal pools, with plants scattered along the roadsides.

Demographic Assessments

In 1992, monitoring methods were developed to rapidly assess the distribution and abundance of *C. palmatum* (CCB 1992). Initially validated on the Livermore population, these methods were sufficient to identify substantial changes in abundance relatively cheaply and quickly. In brief, transects that collectively represented local gradients in land cover and habitat quality were established in four subareas of the site. Each transect was subdivided further into segments representing different vegetation types that were known to support *C. palmatum* or that appeared to be habitat for the species. Observers walked along each transect segment for a predetermined period of time. The number of *C. palmatum* was estimated on a semi-logarithmic scale (none, tens to hundreds, thousands to tens of thousands, and so forth). The number of individuals in each subarea was estimated by summing the totals for each segment.

In 1992 and 1993, populations of *C. palmatum* were surveyed at the Delevan and Colusa National Wildlife Refuges and the Mendota Alkali Sink Ecological Reserve (CCB 1993 unpublished data, CCB 1994). The Woodland population was surveyed in 1992 (CCB 1993 unpublished data). We visited all populations in 2004, and land managers again visited each population in 2012 or 2013. During each visit, biologists familiar with the species estimated population sizes within an order of magnitude on the basis of the rapid-assessment method described above. In addition, refuge managers have conducted annual censuses of the populations at the Delevan and Colusa National Wildlife Refuges since 1997 (J. Silveira unpublished data) and local biologists have conducted annual censuses of the Livermore, Mendota, and Woodland populations (S. Bainbridge unpublished data, E. Cypher unpublished data, C. Little unpublished data).

Genetic Analyses

We sampled 30–35 plants in each of the five extant populations of *C. palmatum* from April to June 2004. We collected two to three short (<5 cm), robust, green branches per plant. The coordinates of each sampled plant were recorded

with a Geoexplorer 3 global positioning system (GPS) meter (Trimble, Sunnyvale, CA). At the Springtown Alkali Sink, we took six samples from a population of *C. molle* subsp. *hispidum* that co-occurred with *C. palmatum* to develop species-specific DNA fragments that could be used to test whether the species hybridize.

We extracted DNA with the proteinase K method detailed in Daehler et al. (1999) at UC Davis. Our preliminary screening of primers identified 28 inter-simple sequence repeat (ISSR) primers (of 100 examined; Nucleic Acid Protein Service Unit Kit #9, University of British Columbia, Vancouver, Canada; <http://naps.msl.ubc.ca>) that amplified *C. palmatum* or *C. molle* subsp. *hispidum* DNA fragments. These are dominant markers that are scored as present or absent. We did not include samples from the Livermore population of *C. palmatum* in the preliminary screening. Instead, we wished to identify species-specific bands for *C. palmatum* from allopatric populations to investigate potential hybridization. We used an Eppendorf Mastercycler Gradient thermocycler to simultaneously optimize $MgCl_2$ and annealing temperature; 17 and 12 primers had annealing temperatures of 50°C and 54°C, respectively. We also screened RAPD (Random Amplified Polymorphic DNA) primers obtained originally from Operon Technology (now known as Qiagen Operon, Inc., Alameda, CA); primers and temperatures are in Appendix 1 (ISSR) and Appendix 2 (RAPD).

We used a final set of 118 bands for our genetic analyses: 86 ISSR bands were polymorphic in *C. palmatum* (69 of these bands were absent in *C. molle* subsp. *hispidum*), 25 ISSR bands were specific to and ubiquitous in *C. molle* subsp. *hispidum*, and 7 bands were specific and ubiquitous in *C. palmatum*. The RAPDs primers were successful for an average of 82% of the samples; as our cut-off value was 90%, they were not used in any further analyses. We used individuals that were scorable for 90% of the DNA bands: 32 individuals of *C. palmatum* from Colusa, 28 from Delevan, 28 from Livermore, 29 from Mendota, and 25 from Woodland (total scored = 142), and 6 individuals of *C. molle* subsp. *hispidum* that were collected from the Livermore site.

PCR conditions were 94°C for 90 sec followed by 40 cycles of 94°C for 15 sec, the optimized annealing temperature for 30 sec, and 72°C for 2 min. Reaction volumes of 15 mL contained, by volume, 10% $MgCl_2$ -free 10X reaction buffer A (Promega, Madison, WI), 0.6 units Taq polymerase (Promega, Madison, WI), 360 picounits primer (University of British Columbia, Vancouver, Canada), 3 mmol/L $MgCl_2$, 200 mmol/L each of dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI), and 30 ng genomic DNA. We repeated most reactions to confirm consistency.

Following electrophoresis on 1.5% agarose gels, we stained DNA with ethidium bromide and visualized the DNA under ultraviolet light. We hand-scored polymorphic DNA fragments in the gels.

Data Analyses

We calculated genetic distance between individuals with a Euclidean distance metric implemented with NTSTSPc (version 2.10 Exeter Software, Setauket, NY). We visualized hierarchical patterns of genetic distance with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method applied to Sequential, Agglomerative, Hierarchical, and Nested (SAHN) cluster analysis. We calculated the geographic distance between individual *C. palmatum*. We calculated and tested the correlation between genetic and geographic distance among and within populations with 1000 permutations of a Mantel test (Mantel 1967). We conducted Mantel tests with the Matrix Comparison Plot program of NTSYS.

We used the program Arlequin 3.0 (Excoffier et al. 1992; <http://lgb.unige.ch/arlequin/>) to calculate a matrix of squared Euclidean genetic distances between all individuals. We included 79 loci (of 86) in the analysis after removing loci with replicate patterns or with >5% missing values. We then partitioned the matrix into submatrices corresponding to subdivisions identified by the UPGMA cluster analysis. The sums of squares in the matrix and submatrices yield sums of squares for the hypothetical divisions in the population. We included the sums of squares in an analysis of molecular variance (AMOVA), which allowed us to test simultaneously whether genetic distances varied between and within groups.

We analyzed both regional genetic structure and genetic differences between *C. palmatum* in two vernal pools within the Colusa National Wildlife Refuge (see above). We calculated a matrix of pairwise differences among populations with Arlequin 3.0. We then applied UPGMA and SAHN cluster analysis to generate a population-level dendrogram of genetic relatedness.

We counted the number of the 86 ISSR polymorphic loci that were variable within each population of *C. palmatum*.

RESULTS

Demographic Assessments

The Mendota Alkali Sink Ecological Reserve contained 800 *C. palmatum* in 1987, but no seedlings or adult plants were observed in the early 1990s. In 2013, 3000 plants were observed (Fig. 1, E. Cypher, unpublished data); presumably these plants germinated from the soil seed

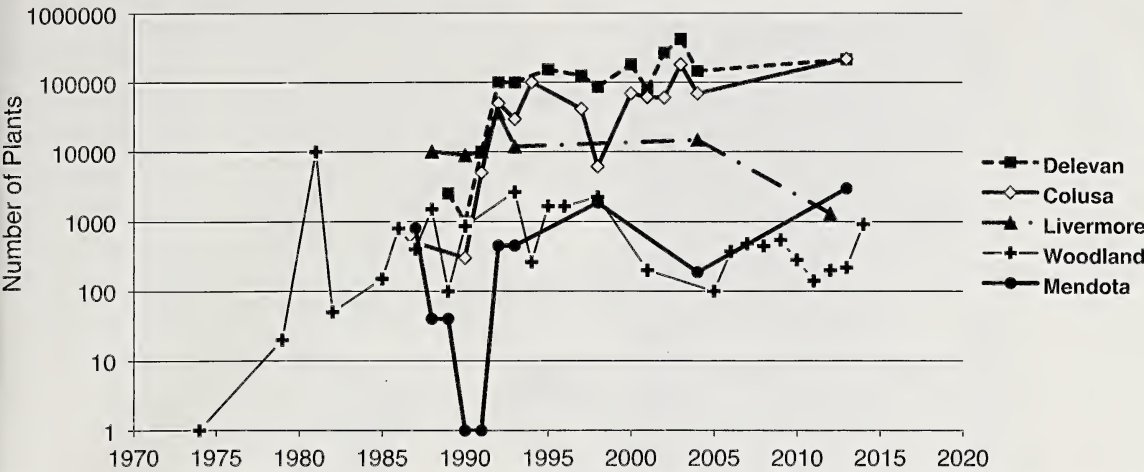


FIG. 1. Changes in abundance of *Chloropyron palmatum* within the five extant populations; note the y-axis is log scale.

bank. Similarly, the Woodland population was nearly extirpated in the mid 1970s. The size of the Woodland population increased to 5000 in 1982, crashed again in the mid 1980s, and has not exceeded a few hundred plants since (Fig. 1, M. Showers and C. Little, unpublished data).

Until recently, annual abundances of *C. palmatum* in the Colusa, Delevan, and Livermore populations ranged from several hundred to many thousands. Since the early 1990s, the number of *C. palmatum* in the Colusa population has fluctuated 3-fold to 7-fold annually; in 2013, more than 200,000 plants were observed at both Colusa and Delevan National Wildlife Refuges (J. Silveira unpublished data, J. Isola unpublished data). For about two decades, the Livermore population was the most stable, with approximately 10,000 individuals, but in 2012 the abundance of *C. palmatum* decreased to 1,100. Multiple factors have been hypothesized to reduce habitat quality at the Springtown Alkali Sink, including altered hydrology, use of off-road vehicles, and invasion by perennial pepperweed (*Lepidium latifolium* L.) and non-native grasses (S. Bainbridge, personal communication).

Genetics

We found no evidence of hybridization between *C. palmatum* and *Chloropyron molle* subsp. *hispidum*. We detected none of the 25 bands specific and ubiquitous to *C. molle* subsp.

hispidum in the Livermore population or in any other population of *C. palmatum*. We did not detect any of the 76 bands specific to *C. palmatum* in *C. molle* subsp. *hispidum*.

Multivariate cluster analysis of genetic similarity between individual *C. palmatum* indicated that the Mendota population was genetically distinct from the four populations to the north (Fig. 2). Individuals from the Delevan and Livermore populations were genetically similar within populations and distinct between populations, whereas the Colusa and Woodland populations were less distinct. In addition to the small, labeled Mendota and Delevan clusters (Fig. 2), 14 individuals were placed in different population clusters than their origin, most especially the individuals collected at Delevan and Mendota. Within populations, we found no correlation between genetic and geographic distance among individuals.

We found no genetic structure between the putative Woodland subpopulations. However, individuals from each vernal pool at Colusa grouped together. In addition, six samples taken from a small roadside aggregation of plants (<20 individuals) at the Delevan National Wildlife Refuge, separated by only 14 linear m from individuals growing in a vernal pool, were genetically distinct from other *C. palmatum* at Delevan (Fig. 2).

On the basis of the hierarchical genetic patterns revealed by multivariate cluster analysis, we

TABLE 1. ANALYSES OF MOLECULAR VARIANCE (AMOVA) AMONG AND WITHIN POPULATIONS OF *CHLOROPYRON PALMATUM*. Potential genetic structure within the Colusa population not included.

Source of variation	df	Sum of squares	Variance	% of variation (P-value)	F _{ST}
Among populations	4	315.74	2.38	22.0 (<0.001)	
Within populations	143	1208.3	8.45	78.0 (<0.001)	
					0.22 (<0.001)

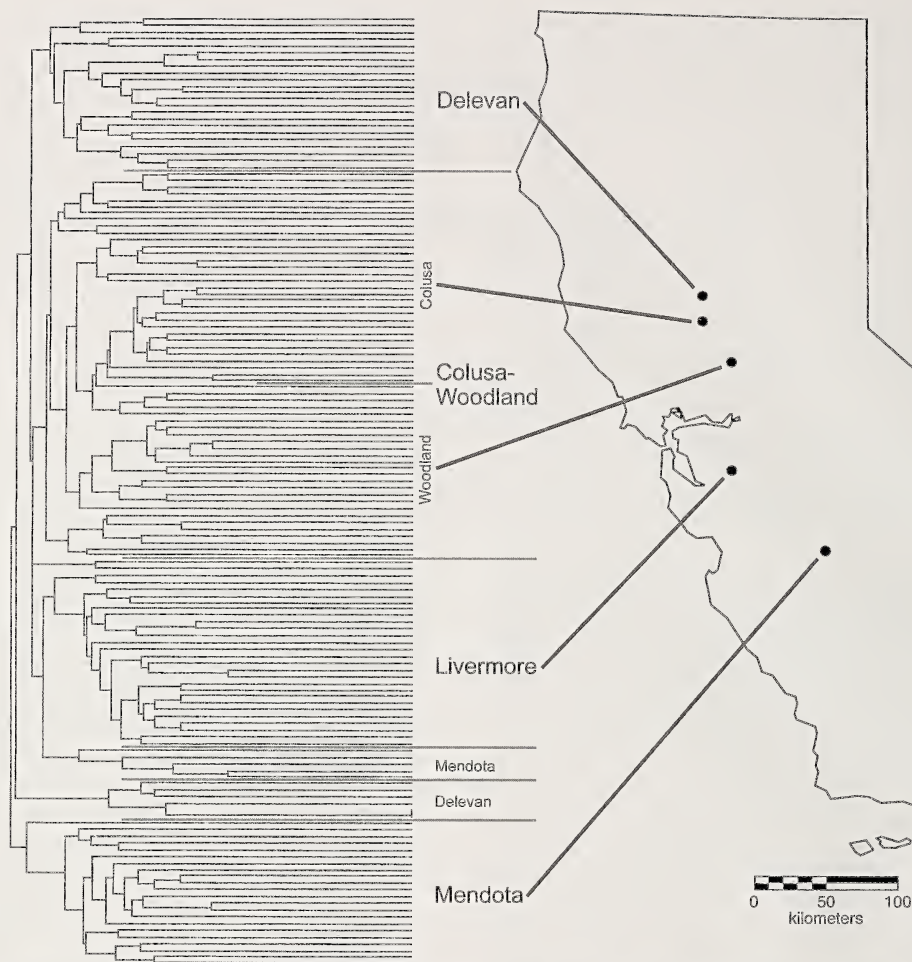


FIG. 2. Individual *Chloropyron palmatum* clustered on the basis of genetic similarity and the location of populations within California, USA.

performed two AMOVA. The first AMOVA did not account for potential differences among putative subpopulations (Table 1), whereas the second AMOVA incorporated potential differences between individuals from two vernal pools at Colusa National Wildlife Refuge (Table 2). In both analyses, 76–78% of genetic variation occurred within populations and the remaining genetic variation occurred among populations or subpopulations, resulting in similar F_{ST} values of ca. 0.22 ($P < 0.001$). In the second analysis,

almost 10% of among-population variation was attributed to subpopulation structure at Colusa. Cluster analysis of pairwise differences between populations indicated division between northern and southern populations (Fig. 3). The dendrogram based on genetic similarity among individuals suggested that the Livermore population was more closely related to the northern than the southern populations. However, the population-level analysis suggested that the Livermore population was most closely related

TABLE 2. ANALYSES OF MOLECULAR VARIANCE (AMOVA) AMONG AND WITHIN POPULATIONS OF *CHLOROPYRON PALMATUM*. Potential genetic structure within the Colusa population included.

Source of variation	df	Sum of squares	Variance	% of variation (P-value)	F_{ST}
Among populations	4	315.74	1.44	13.28 (0.067)	
Among putative subpopulations	1	25.9	1.06	9.84 (<0.001)	
Within populations	143	1182	8.33	76.89 (<0.001)	
					0.23 (<0.001)

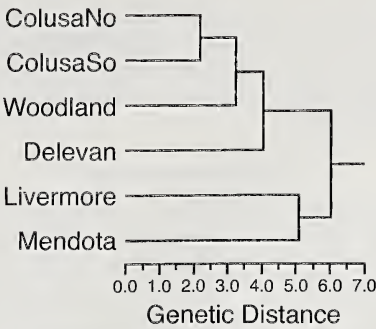


FIG. 3. Populations of *Chloropyron palmatum* clustered on the basis of genetic similarity; ColusaNo and ColusaSo are two adjacent vernal pools separated by 120 m.

to, although genetically distant from, the southernmost population, Mendota. The genetic structure of the northern population group generated by the population-level analysis was consistent with that from the individual-based dendrogram.

Populations of *C. palmatum* contained an average of 59 ± 3 (mean $\pm$ SD) of the species' 86 polymorphic loci (Mendota and Livermore, 56; Colusa, 58; Delevan, 61; Woodland, 63).

DISCUSSION

Conservation efforts generally aim to maintain not only distribution and abundance of a given species but its evolutionary potential (Hufford and Mazer 2004). Conservation of evolutionary potential requires both an assessment of genetic structure and an evaluation of potential mechanisms that create genetic structure. We employed neutral genetic markers to assess gene flow and genetic variation in *C. palmatum* and evaluated the mechanisms by which gene flow might occur. However, we did not assess genetic structure that may reflect environmental adaptation.

Gene flow in plants occurs either by movement of haploid gametes in pollen grains or by seed dispersal. We found that geographic and genetic definitions of populations of *C. palmatum* were similar, suggesting that populations have been isolated for many generations. Historically, thousands of acres of the Colusa Basin from Knight's Landing to Willows were alkali flats, vernal lakes and pools that were eventually converted to rice acreage (Silviera 2000), fragmenting what may have been a vast ancestral population of *C. palmatum*, to the current isolated remnant populations at Delevan, Colusa, and Woodland. This would explain their close genetic affinities, especially between the Colusa and Woodland populations (Fig. 2). It is likely that the Livermore population, hemmed in by hills, and the Mendota population, 290 km distant from Woodland were always isolated. There was no

apparent genetic structure within the Mendota, Livermore, and Woodland populations. In contrast, individuals in two adjacent vernal pools at Colusa were genetically distinct. Although we did not detect structure within the Delevan population, our sample sizes from several vernal pools were insufficient to be confident that no genetic differences exist. Genetic structure at the level of vernal pools has been considered as a theoretical possibility (e.g., Elam 1998). Recently, Gordon et al (2012), using microsatellites, found genetic structure among vernal pools within locations in *Neostapfia colusana* (Burt Davy) Burt Davy ($F_{ST} = 2.73$) and *Tuctoria greenei* (Vasey) Reeder ($F_{ST} = 7.4$).

Early genetic analyses of species that inhabit vernal pools examined species-level genetic structure based on isozyme variation (Crawford and Ornduff 1989, Dole and Sun 1992). A challenge in basing genetic inferences on isozymes, especially for rare species, is that the enzymes are not sufficiently variable to allow robust analysis of population structure. For example, six of nine putative populations of *Limnanthes floccosa* Howell subsp. *californica* Arroyo were invariant at 28 isozyme loci, and variable populations were polymorphic at but a single locus (Dole and Sun 1992). By contrast, a DNA-based study of these populations found that over 50% of 457 plants had unique genetic profiles, and identified 20 genetically distinct populations (Sloop et al. 2011).

Previous genetic analyses of *C. palmatum* that were based on isozyme alleles (Fleishman et al. 2001) yielded an F_{ST} of 0.019, less than 10% of the genetic structure we detected with nuclear DNA markers. In addition, previous work suggested that the large populations at Delevan and Colusa National Wildlife Refuges were virtually invariant, whereas we found that almost 60% of ISSR loci within these populations were variable. However, our estimate of total variation was affected by our use of polymorphic DNA markers. Both the allozyme and the DNA analyses found that genetic variability was not correlated with population size. We believe that the isozyme analysis did not contain enough alleles to discriminate adequately among genotypes and therefore underestimated genetic divergence.

It appears that relatively small populations of *C. palmatum*, even those with a history of severe bottlenecks, can contain relatively high levels of genetic variation. Past differences in abundance between the Mendota and Livermore populations notwithstanding, the number of polymorphic loci in the two populations was the same. Despite two recent population bottlenecks, the small Woodland population contained the greatest number of polymorphic loci, comparable to the much larger population at Delevan. Maintenance of genetically variable populations regardless of

demographic stochasticity is likely attributable to a genetically variable soil seed bank. In the endangered vernal pool endemic *Limnanthes vincularis* Ornduff, temporal genetic structure within a given vernal pool varied substantially among years, indicating that single-year estimates may not reflect the genetic variation retained in the soil seed bank (Sloop et al. 2012).

Our results suggest that hydrologic connectivity may have a considerable effect on genetic structure within populations and that dispersal of buoyant seeds has facilitated long-term gene flow in *C. palmatum*. For example, lack of genetic structure within the Livermore and Woodland populations may reflect historical or recent overland flow. By contrast, genetic differences between individuals in adjacent vernal pools at the Colusa National Wildlife Refuge suggest that overland flow between the pools is infrequent.

Application of Results to Conservation

Our work suggests that conservation efforts for *C. palmatum* are most likely to be effective when historical patterns of hydrology are maintained. We suggest maintaining both hydrologic barriers in populations with genetic substructure and overland flow in populations without local genetic substructure.

If historical patterns of overland water flow cannot be maintained, then mixing of seed collected from throughout a given population may mimic historical exchange of genes within that population. However, mixing seed may disrupt locally coadapted gene complexes or generate outbreeding depression (Hufford and Mazer 2003, but see Frankham et al. 2010). There is considerable evidence that local adaptation strongly affects genetic structure in plants (Linhart and Grant 1996); in most cases, there appears to be no correlation between neutral genetic markers and morphological traits (McKay and Latta 2002). Accordingly, genetic structure that is driven by gene flow and revealed by selectively neutral molecular markers may not correspond with morphological patterns driven by long-term environmental adaptation. Moreover, mitigation efforts that sow seeds across a relatively large area may lead to genetic homogenization, possibly reducing microevolutionary potential (Halbur et al. 2014).

Because all populations of *C. palmatum* are genetically variable and distinct, we recommend that seed collection and sowing be conducted within rather than among populations. As well, seed collections should be made over multiple years to capture the genetic variation in the seed bank. On the basis of our work, we suggest that sowing and collection of seeds reflect historical hydrology, and that populations that may be hydrologically isolated, such as those within

vernal pools, be maintained intact. Where human activities have impeded overland flows, site-wide sowing of seed collected from throughout the population may simulate historical patterns of seed movement.

ACKNOWLEDGMENTS

We thank the *Chloropyron palmatum* consortium, especially Mary Ann Showers, Joe Silviera, Jennifer Isola, Ellen Cypher, Sue Bainbridge, Cathy Little, and the Center for Natural Lands Management for data and insightful discussions, and are grateful for the comments of three reviewers. Funding for this project was provided by the California Department of Fish and Game under federal Section 6 Grant Number E-2-P-23; an earlier report was posted on their website (Ayres et al. 2007). Collections were conducted under federal permit TE 083783 to DA.

LITERATURE CITED

- ALLEN, W. H. 1994. Reintroduction of endangered plants: biologists worry that mitigation may be considered an easy option in the political and legal frameworks of conservation. *Bioscience* 44:65–68.
- AYRES, D. A., E. FLEISHMAN, A. LAUNER, A. LEE, AND D. ZIPPIN. 2007. Development of demographic and genetic conservation strategies for maintenance of an endangered annual plant. Unpublished report. Website <https://nrm.dfg.ca.gov/FileHandler.ashx?DocumentID=23328&inline=1> (accessed 02 March 2015).
- CENTER FOR CONSERVATION BIOLOGY (CCB). 1992. Studies of the *Cordylanthus palmatus* at the Springtown Alkali Sink, Livermore, California. Website <https://nrm.dfg.ca.gov/FileHandler.ashx?DocumentID=3207> (accessed 18 November 2014).
- . 1994. Conservation of the palmate-bracted bird's-beak (*Cordylanthus palmatus*). Website <https://nrm.dfg.ca.gov/FileHandler.ashx?DocumentID=3126> (accessed 18 November 2014).
- CHUANG, T. I. AND L. R. HECKARD. 1971. Observations on root-parasitism in *Cordylanthus* (Scrophulariaceae). *American Journal of Botany* 58:218–228.
- . 1972. Seed coat morphology in *Cordylanthus* (Scrophulariaceae) and its taxonomic significance. *American Journal of Botany* 59:258–265.
- CRAWFORD, D. J. AND R. ORNDUFF. 1989. Enzyme electrophoresis and evolutionary relationships among three species of *Lasthenia* (Asteraceae: Heliantheae). *American Journal of Botany* 76:289–296.
- DAEHLER, C. C., C. K. ANTILLA, D. R. AYRES, AND D. R. STRONG. 1999. Evolution of a new ecotype of *Spartina alterniflora* (Poaceae) in San Francisco Bay, California, USA. *American Journal of Botany* 86:543–546.
- DOLE, J. A. AND M. SUN. 1992. Field and genetic survey of the endangered Butte County meadow-foam - *Limnanthes floccosa* subsp. *californica* (Limnanthaceae). *Conservation Biology* 6:549–558.
- ELAM, D. 1998. Population genetics of vernal pool plants: theory, data and conservation implications. Pp. 180–189 in C. W. Witham et al. (eds.), *Ecology, conservation, and management of vernal pool ecosystems: proceedings from a 1996 conference*. California Native Plant Society, Sacramento, CA.

EXCOFFIER, L., P. E. SMOUSE, AND J. M. QUATTRO. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131:479–491.

FLEISHMAN, E., A. E. LAUNER, K. R. SWITKY, U. YANDELL, J. HEYWOOD, AND D. D. MURPHY. 2001. Rules and exceptions in conservation genetics: genetic assessment of the endangered plant *Cordylanthus palmatus* and its implications for management planning. *Biological Conservation* 98:45–53.

FOIN, T. C., S. P. REILLY, A. L. PAWLEY, D. R. AYRES, T. M. CARLSON, P. J. HODEM, AND P. V. SWITZER. 1998. Improving recovery planning for the conservation of threatened and endangered taxa. *Bioscience* 48:177–184.

FRANKHAM, R. 2010. Challenges and opportunities of genetic approaches to biological conservation. *Biological Conservation* 143:1919–1927.

GITZENDANNER, M. A. AND P. S. SOLTIS. 2000. Patterns of genetic variation in rare and widespread plant congeners. *American Journal of Botany* 87:783–792.

GORDON, S. P., C. M. SLOOP, H. G. DAVIS, AND J. H. CUSHMAN. 2012. Population genetic diversity and structure of two rare vernal pool grasses in central California. *Conservation Genetics* 13: 117–130.

HALBUR, M. M., C. M. SLOOP, M. J. ZANIS, AND N. C. EMORY. 2014. The population biology of mitigation: impacts of habitat creation on an endangered plant species. *Conservation Genetics* 15:679–695.

HAMRICK, J. L. AND M. J. W. GODT. 1989. Allozyme diversity in plant species. Pp. 43–63 in A. H. D. Brown, M. T. Clegg, A. L. Kahler, and B. S. Weir (eds.), *Plant population genetics, breeding, and genetic resources*. Sinauer, Sunderland, MA.

HOWALD, A. M. 1996. Translocation as a mitigation strategy: lessons from California. Pp. 293–330 in D. A. Falk, C. I. Millar, and M. Olwell (eds.), *Restoring diversity: strategies for reintroduction of endangered plants*. Island Press, Washington, D.C.

HOWE, H. F. AND J. SMALLWOOD. 1982. Ecology of seed dispersal. *Annual Review of Ecology and Systematics* 13:201–228.

HUFFORD, K. H. AND S. J. MAZER. 2003. Plant ecotypes: genetic differentiation in the age of ecological restoration. *Trends in Ecology and Evolution* 18:147–155.

IWANAMI, Y., T. SASAKUMA, AND Y. YAMADA. 1998. *Pollen: illustrations and scanning electron micrographs*. Kodansha, Tokyo, Japan.

LEE, L. C. AND ASSOCIATES. 2002. The palmate bird's beak at the Springtown Alkali Sink Wetlands, Livermore Valley, California: rapid assessment of *Cordylanthus palmatus* flower visitors. L.C. Lee and Associates, Alameda, CA and Center for Conservation Biology, Stanford University, Stanford, CA.

LINHART, Y. B. AND M. C. GRANT. 1996. Evolutionary significance of local genetic differentiation in plants. *Annual Review of Ecology and Systematics* 27:237–277.

MALIAKAL-WITT, S., E. S. MENGES, AND J. S. DENSLOW. 2005. Microhabitat distribution of two Florida scrub endemic plants in comparison to their habitat-generalist congeners. *American Journal of Botany* 92:411–421.

MANTEL, N. 1967. The detection of disease clustering and a generalized regression approach. *Cancer Research* 27:209–220.

MCKAY, J. K. AND R. G. LATTA. 2002. Adaptive population divergence: markers, QTL and traits. *Trends in Ecology and Evolution* 17:285–291.

PROCTOR, M. AND P. YEO. 1972. *The pollination of flowers*. Taplinger, New York, NY.

RAMP, J. M., S. K. COLLINGE, AND T. A. RANKER. 2006. Restoration genetics of the vernal pool endemic *Lasthenia conjugens* (Asteraceae). *Conservation Genetics* 7:631–649.

SILIERA, J. G. 2000. Alkali vernal pools at Sacramento National Wildlife Refuge. *Fremontia* 27:10–18.

SLOOP, C. M., R. EBERL, AND D. R. AYRES. 2012. Genetic diversity and structure in the annual vernal pool endemic *Limnanthes vincularis* Ornduff (Limnanthaceae): implications of breeding system and restoration practices. *Conservation Genetics* 13:1365–1379.

———, C. PICKENS, AND C. P. GORDON. 2011. Conservation genetics of Butte County meadow-foam (*Limnanthes floccosa* subsp. *californica* Arroyo), an endangered vernal pool endemic. *Conservation Genetics* 12:311–323.

U.S. FISH AND WILDLIFE SERVICE (USFWS). 1998. *Recovery plan for upland species of the San Joaquin Valley, California*. Portland, OR.

APPENDIX 1. ISSR PRIMERS USED IN STUDY. An asterisk (*) indicate a *Chloropyron molle* subsp. *hispidum* specific band. A superscript caret (^) indicates a *Chloropyron palmatum* specific band.

Primer	Annealing temp °C	Informative bands (bp)					Sequence 5'–3'	
807	54	330	370^	475	700	825	900	AGAGAGAGAGAGAGT
809	50	525*	550	730	830	870*		AGAGAGAGAGAGAGG
810	54	500	620*	900	950			GAGAGAGAGAGAGAT
811	54	400	790					GAGAGAGAGAGAGAC
818	54	580	720	770	900	1050	2000*	CACACACACACACAG
823	54	550	1000	1270*	2000			TCTCTCTCTCTCTCC
825	50	770	800	840	1900			ACACACACACACACT
826	50	590	610*	670*	710	1130*	1300	ACACACACACACACC
827	54	510	530*	600	770	1050	1400	ACACACACACACACG
834	54	900	2000				2000	AGAGAGAGAGAGAGYT
835	50	470	610					AGAGAGAGAGAGAGYC
836	54	870	1150*	1600	1750			AGAGAGAGAGAGAGYA
841	50	410^	1200	1800				GAGAGAGAGAGAGAYC
844	50	1170						CTCTCTCTCTCTCTRC
848	54	230	310	560	720	870^	925	CACACACACACACARG
849	50	1050	1160	1900*	2100*		1460	GTGTGTGTGTGTGTGTYA
855	50	550	1200	1230*	1370			ACACACACACACACACYT
856	50	650^	775	830*	1200*			ACACACACACACACACYA
857	50	470	700*	920	1100	1200		ACACACACACACACACYG
860	50	600^	700*	900*	1270^	1500		TGTGTGTGTGTGTGTGRA
876	50	520	770	1250				GATAGATAGACAGACA
884	50	600	660	830	870			HBHAGAGAGAGAGAGAG
885	54	530	660	700	950			BHBGAGAGAGAGAGAGA
886	54	440*	525	757	1150	1200	1400	VDVCTCTCTCTCTCT
887	54	530	570	1000	1200			DVDTCTCTCTCTCTC
888	50	1070	1130*					BDBCACACACACACACA
889	50	750*	790^	810*	925*	1650*		DBDACACACACACAC
899	50	575	850					CATGTTGTTGGTCATTGTTCCA

APPENDIX 2. RAPD PRIMERS SCREENED IN STUDY.

Primer	Annealing temp. °C		Informative bands (base pairs)				Sequence 5'–3'
A04	42	570					AATCGGGCTG
A10	42	700	800	1600			GTGATCGCAG
A11	42	1500					CAATCGCCGT
B06	42	570	700	850	900	950	TGCTCTGCCC
B10	42	600	650				CTGCTGGGAC
F06	42	325	700				GGGAATTCCG
G03	42	560	700				GAGCCCTCCA
G18	42	850					GGCTCATGTG
H02	42	650	700				TCGGACGTGA
H03	42	750	900				AGACGTCCAC
H04	42	750	800				GGAAGTCGCC
H19	42	1300					CTCGACCAGCC
212	42	625					GCTGCGTGAC